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A molecular characterization of the fungal endophytes within the needles of ponderosa pine (Pinus ponderosa)

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A MOLECULAR CHARACTERIZATION OF THE FUNGAL ENDOPHYTES WITHIN THE NEEDLES OF PONDEROSA PINE (*PINUS PONDEROSA*)

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MASTER'S THESIS

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ABSTRACT

Fungal endophytes (microbial fungi that live in plant tissues without causing disease) are ubiquitous in plants studied to date. Many conifers host endophytes that produce important bioactive secondary metabolites. While the endophytes in *Pinus ponderosa* roots have been studied, the endophyte communities within the needles are not well characterized by molecular means: thus, the goal of this study was to do so. Needles were sampled at two sites located five kilometers apart along a slight precipitation gradient in Eastern Washington State. Fungal DNA (along with pine DNA) was isolated directly from needles of two ages. Internal transcribed spacer regions of the ribosomal RNA gene form fungi were amplified by PCR, cloned, and sequenced. Many of the sequences described have high homology matches in GenBank with known fungi, though most have not previously been directly associated with ponderosa pine. Some sequences were highly homologous with previously published sequences of fungi that have yet to be assigned a taxonomic designation , which suggests some previously undescribed fungi occupy ponderosa pine needles. Despite the proximity of sites, only 18% of the endophytes sequenced were common to both sites. Forty one percent of endophytes were unique to needles from 2012, while 45% of endophytes were unique to needles that emerged in 2014. Although more data from a deep sequencing project will be required to confirm this, the results presented here suggests that fungal endophyte communities in ponderosa pine are fairly diverse geographically, and that communities in the same tree differ depending on the age of the needles.

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INTRODUCTION

The kingdom Fungi is remarkably rich, with more than 100,000 described species, and as many as five million projected undescribed species (BLACKWELL 2011; GAMBOA *et al.* 2002; TOJU *et al.* 2012). Fungi range from the largest known organism on Earth, to microbial elements of soil, terrestrial and aquatic ecosystems (CLAY 1988). They exist as saprophytes and colonize both plans and animals. Fungi produce a wealth of enzymes that break down cellulose, proteins, lignin, and chitin into absorbable components (BOYLE *et al.* 1992; MARTINEZ *et al.* 2009). Fungal interactions with plants span the spectrum from mutualistic associations that are obligate for plant survival through apparently commensal relations to lethal pathogenic infections.

Plant/fungi interactions

Most vascular plants have mutualistic mycorrhizal relationships with fungi (WANG and QIU 2006), and arbuscular mycorrhizae can be clearly observed in fossil samples of the earliest known vascular plants from the Rhynie chert bed in Scotland (REMY *et al.* 1994). Pathogenic interactions between plants and fungi are also well documented (GRZYWACZ and WAZNY 1973; KNOGGE 1996; VON SCHÜTT 1971). While some calculations suggest that fewer than 10% of known fungi are capable of inhabiting living plant tissue, all plant species are attacked by pathogenic fungi (KNOGGE 1996). Fungal pathogenicity in plants is so widespread that it is nearly impossible to calculate world-wide annual pre- and post-harvest total crop losses due to fungal pathogens. A single fungus, *Magnaporthe oryzae*, is responsible for up to 18% of annual rice crop losses (SOANES *et al.* 2012), at a cost of $66 billion of market value.

Fungi also exist as endophytes within plants. Fungal endophytes are fungi that exist within the leaf, stem, and/or root tissues of plants but lack the extra-matrical hyphae characteristics of mycorrhizal fungi, and do not cause apparent disease symptoms (CARROLL 1988; CLAY 1988). This is a long-suspected interaction that has been difficult to observe and

challenging to characterize (CLAY 1988). Endophytic functions within plant systems are still being explored: in most cases, it appears that host plants benefit from their presence (ALY *et al.* 2011; QADRI *et al.* 2013; STROBEL *et al.* 2008; SURYANARAYANAN *et al.* 2012).

The darnel – a weedy grass that has a tendency to grow among cereal crops – was the first plant with documented entophytic interactions (FREEMAN 1904). This grass is moderately toxic when consumed by herbivores, which led to an investigation as to what was causing toxicity (DUTTA *et al.* 2014). The grass, it turns out, hosts microbial fungi that confer herbivore deterrence. Other frequently grazed plants also host fungal endophytes that have been shown to confer herbivory deterrence. Fescue is a common perennial forage that periodically causes problems for large grazers. Cows, horses, and sheep grazing in endophyte-hosting pastures may be afflicted with palsies and hoof lesions. Ryegrass staggers is another infamous seasonal grazing issue. Large herbivores consuming endophyte-infected ryegrass forage may experience tremors, stiffened limbs, and loss of coordination (RICHARDSON 2000). Both of the latter conditions are usually recoverable; however, animals may require several months of convalescence to return to normal function.

Fungal endophytes also can provide their hosts with some protection from herbivorous insects; which, if that property is properly harnessed, may allow for reduced pesticide application. Thrip herbivory reduces Kenyan onion crop yields by as much as 59% annually, and the cryptic nature of thrip feeding (the insects nestle between sheathes of the onion leaves) often requires heavy insecticide applications (MUVEA *et al.* 2014). However, onion seeds inoculated with fungal endophytes extracted from healthy onion crops produced plants with fewer thrips, feeding punctures, and eggs; additionally, total onion crop yield increased significantly (MUVEA *et al.* 2014). Potential uses for endophytes as biocontrol agents have spurred research into

inoculation of tuber-producing plants to reduce insect, nematode, and fungal damage (ZHANG *et al.* 2014).

Endophytic fungi also can confer plants with tolerance to abiotic stresses (KRANNER *et al.* 2005; SWARTHOUT *et al.* 2009). A panic grass (*Dichanthelium lanuginosum*) that grows in Yellowstone National Park thrives in hot soils with temperatures upward of 50°C. If the grass is "cured" of its fungal endophyte, the grass is no longer able to withstand high soil temperatures (MARQUEZ *et al.* 2007). Tomato plants inoculated with the same endophyte were able to thrive in increased soil temperatures, while the control plants failed. Interestingly, it is a mycovirus *hosted* by the fungal endophyte that confers the resistance to increased soil temperatures. Plants cannot be infected with the virus alone and the thermotolerance only is conferred when the virus is hosted within the endophyte (MARQUEZ *et al.* 2007).

In a stress resistance study with barley, researchers determined that an endophyte cultivated from a wild barley strain confers increased salt tolerance to commercial barley strains (WALLER *et al.* 2005). A series of hydroponic tests in the same study showed that inoculated commercial strains produced more seeds per head, and also developed some resistance to root rot (WALLER *et al.* 2005).

Some normally mutualistic or commensal endophytes may change from a mutualistic to a pathogenic state in a host if environmental conditions favor the shift (ARNOLD 2007; SCHULZ and BOYLE 2005). If some endophytes are, in fact, latent pathogens, it is possible that an endophyte-host mutualism may become a pathogen-host relationship if the host is subjected to prolonged biotic or abiotic stress (SCHULZ and BOYLE 2005). *Phomopsis viticola* in grape leaves (*Vitis vinifera*), *Rhabdocline parkeri* in Douglas fir (*Pseudotsuga menziesii*), and *Lophodermium spp.* in western white pine (*Pinus strobus*) are all fungal endophytes found to exist asymptomatically in their hosts. If *P. viticola* is extracted, grown in isolation in the lab, and applied to the surfaces

of the leaves in which the fungus resides, the result is tissue death and/or leaf senescence (HYDE and SOYTONG 2008). Similarly, the endophyte *R. parkeri* exists as a saprobe in epidermal cells of fir needles, but begins growth after needle senescence (STONE 1987). *Lophodermium* species in *Pinus strobus* transition to a pathogenic life cycle during host senescence (SCHULZ and BOYLE 2005). *Lophodermium* blights are endemic, and sometimes inconspicuous, but periodically produce widespread outbreaks.

Endophyte-produced compounds

Fungal endophytes produce a number of different compounds- alkaloids, terpenoids, steroids, phenolics, isocoumarins, and volatile organic compounds, some of which have potential applications within the field of medicine (DAISY *et al.* 2002). One of the best-studied examples of a medicinally valuable compound from endophytes is Taxol, originally extracted from the Pacific yew (*Taxus brevifolia*, WANI *et al.* 1971; ZHANG *et al.* 2009; ZHAO *et al.* 2009). The compound acts as a mitotic inhibitor, and has been used extensively as a chemotherapy agent (LI *et al.* 1996; LONG and FAIRCHILD 1994). In 1993, Taxol production was shown in both the tree's bark and by fungal endophytes in the needles. This was the first demonstration of an anti-cancer chemical being produced by a fungal endophyte (STROBEL and DAISY 2003; ZHOU *et al.* 2010).

Taxol's utility stimulated further investigation into plant-endophyte interactions (KUMARAN *et al.* 2011). These investigations have led to the isolation of endophytes that may prove useful in the battles against diabetes, rheumatoid arthritis, and antibiotic resistance (STROBEL and DAISY 2003). Additionally, the antioxidant properties demonstrated by some endophytes may assist in diminishing the damage of hypoxia and reperfusion experienced by patients suffering stroke or cardiac arrest (HUANG *et al.* 2007; TIANPANICH *et al.* 2011). The search for new fungal endophytes continues. They have been isolated from every plant examined, and are likely to inhabit every plant on Earth (ARNOLD *et al.* 2003; MCCUTCHEON *et*

al. 1993; SINGH *et al.* 2011). However, characterizing the species compositions of endophyte communities within plant tissues has proven a challenge.

Describing endophyte communities

Culturing is the tradition and most common approach for fungal endophyte discovery; however, this method has limitations. First, samples from one plant species (or even one individual) might yield cultures in varying states of maturity that appear different, but are actually the same fungal species. Such results have made it difficult to establish whether an endophytic community differs from leaf to leaf, or whether the differences are an artifact of culturing technique (PERSOH 2013; RAJALA *et al.* 2013). For example, leaf fragment size is inversely correlated with the number of endophytes emerging on plated media (GAMBOA *et al.* 2002). Another challenge is the relative fastidiousness of many fungi in a lab environment. Predicting appropriate nutrients to encourage fungal emergence from plant tissue onto plated media is notoriously tricky, as is determining optimal humidity, temperature, and light conditions for growth (KIM *et al.* 2013; SUN and GUO 2012). Additionally, it is common to culture fungal hyphae that cannot be induced to produce identifiable spores which are essential for successful identification. There are no current reliable methods of morphological species identification by hyphae alone. Given these challenges, DNA isolation and sequencing is now being used to detect and identify endophytes. As molecular technology improves (both in reach and in price), endophyte studies are shifting from culturing techniques toward direct molecular evaluation of endophytes (BAEK and KENERLEY 1998). As an example, culture-dependent methods revealed 29 fungal endophytes in grape leaves while molecular methods revealed 39 (GRISAN *et al.* 2011).

Given that every plant species examined so far has endophytes, and given that at present it is unfeasible to test every known plant, we are left with the difficult task of choosing which plant species should be examined for endophyte communities. One approach might be to test

plants that are very common, widely distributed, and/or related to plants that have yielded useful endophyte products in the past (e.g. Taxol from the endophytes in Pacific Yew). Ponderosa pine (*Pinus ponderosa*) fits all of these parameters. Like Pacific yew, Ponderosa pine is a conifer plus it occupies the widest range of any pine tree in North America. It is found at elevations ranging from sea level to 3050 meters, extends from British Columbia, Canada through San Luis Potosí, Mexico; and occupies 16 US states in between (BURNS and HONKALA 1990).

The tree's large distribution means it is exposed to an array of biotic and abiotic stresses. Because of ponderosa pine's range and abundance, we have only just begun to explore the diversity of fungal endophytes within the needle community of the tree. A single culture-based study has explored the endophyte community of ponderosa pine at a single location in the University of Idaho Experimental Forest (Boise, ID). The most common endophytes recovered from healthy needles were *Lophodermium* species, *Sydowia polyspora, Elytroderma*, *Cladosporium*, and *Penicillium* species (RIDOUT and NEWCOMBE 2015). These isolates were used individually to inoculate newly emerging needles in a stand of trees where *Dothistroma* needle blight had been identified to determine whether those endophytes could modify disease severity, and showed that they did (RIDOUT and NEWCOMBE 2015). As previous work concerning ponderosa needle endophytes has been limited to one sample location, we know nothing of diversity of endophytes at different geographic locations, among trees at the same location or among needles on the same tree.

The goals of this study were to expand our knowledge of endophyte communities within Ponderosa pine at different locations, and to determine whether endophyte communities vary within trees depending on the ages of the needles sampled. To achieve these goals I answered three questions. First, would molecular techniques reveal a larger community of fungi than could be detected with standard cultivation techniques? Second, would there be any difference in the

needles communities between sampling sites? Third, would there be a difference in community composition in needles of different ages?

MATERIALS AND METHODS

Sampling site description

Needles were collected in March 2015. Two local sites in southwest Spokane County, WA that had similarly-aged stands of *P. ponderosa* were chosen. At each site blight had left a uniform absence of needles that had emerged in 2013 (Figure 1). Ten trees were selected for sampling at each site. The first site, at Tyler, WA (GPS coordinates 47.436111, -117.783611), is open woodland with interspersed dwellings on 3-5 ha parcels. The understory consists of low shrubs (primarily *Symphoricarpos albus* and *Rosa woodsii*), and bunch grasses (*Pseudoroegneria spicata* and *Festuca idahoensis*). This general community type, interspersed with meadows and small wetlands, exists for several kilometers in all directions from the sample site. The second, at Hog Lake, WA (GPS coordinates 47.3800, -117.783611), is at the border of an ecotone where pine gives way to Columbia Basin sagebrush-steppe dominated by threetip sagebrush (*Artemesia tripartita).* The understory includes some *Symphoricarpos albus*, and a heavier presence of grasses (*Pseudoroegneria spicata* and *Festuca idahoensis*). There are no dwellings in the immediate vicinity of the second site, but the area is crisscrossed with hiking trails.

Endophyte culturing

Healthy needles were collected at each site from each of 10 trees at breast height. Needles were bagged, labeled, stored at 4°C, and processed for culturing within forty-eight hours. Each needle was examined for visible signs of fungal infection and insect damage, and needles with blemishes or damage were discarded. Unblemished needles were surface-sterilized by immersion in 70% isopropyl alcohol for 30 seconds, rinsing with distilled water, and re-immersion in

isopropanol for 30 seconds prior to a final rinse in autoclaved distilled water. After surface sterilization needles were crushed in a sterile mortar and pestle. In preliminary attempts to culture endophytes from needles, compressing needles in a sterile mortar and pestle after surface sterilization increased the number of emerging fungal colonies three-fold. This tissue disruption step was subsequently incorporated into my standard plating protocol. When flattened enough to increase the surface area of needle tissue in contact with the media, the needles were sectioned. Eight needle sections (2 cm in length) were plated on V8 media (400 mL distilled H2O, 100 mL V8, 1 g $CaCO_3$, 7.5 g agar/500 mL preparation) with 50 µg/mL ampicillin added to discourage bacterial growth. One hundred and twenty-five plates were made for each needle age at both sites, for a total of 500 plates at the conclusion of the plate culturing process.

Plates were incubated at room temperature (RT) with direct exposure to natural light and dark cycles. Culturing was conducted over a period of eight months from April to December 2015. Endophytes emerged between four and 15 days after plating. Fungi emerging with distinctive morphologies were transferred to fresh plates and cultured in isolation for up to two months to allow for the possibility of spore formation. Both fungi forming spores and isolates that did not form spores were examined under the microscope for hyphal structure.

DNA extraction

Forty-eight healthy needles were collected at each site from each of 10 trees at breast height (twenty-four 2012 needles, and twenty-four 2014 needles); needles were bagged, labeled, stored at 4°C, and processed for DNA purifications within forty-eight hours. For each age class of needles, samples from the ten trees were pooled for each site. Needles were surface-sterilized in ethanol to prevent the extraction of DNA from epiphytic fungi. DNA extraction procedures were performed following a modified cetyltrimethylammoniumbromide, or "CTAB" (TELFER *et al.* 2013). A CTAB (MP BIO) working solution ($(C_{16}H_{33})N(CH_3)_3Br$) was prepared (TELFER *et al.*

2013) (See Appendix 1 for detail). Chopped needle tissue (250 mg) was homogenized in liquid nitrogen with a mortar and pestle until a fine powder was achieved, and the powder was transferred while frozen to 1.5 mL polyethylene screw-cap grinding vials with 25 (0.5 mm diameter) glass beads and 600 µL of warm (65°C) CTAB buffer. Needle tissue was further homogenized in a Micro mini-bead beater (BioSpec Model 1) for 60 seconds at 3400 strokes per minute. After a 60 minute incubation at 65°C, tubes were centrifuged at room temperature for 10 minutes at 2000 g; the resulting supernatant (roughly 500 µL) was transferred to a 1.5 mL Eppendorf tube. The solution was stabilized with 250 µL of 5 M NaCl (J.T. Baker), and 200 µL of 24:1 chloroform:isoamyl (ScholAR Chemistry) was added and mixed by inversion. Following a 20-minute centrifugation at 18000 g, the aqueous layer was removed to a fresh tube. After a second extraction with 400 µL of 24:1 chloroform:isoamyl, samples were centrifuged for another 20 minutes at 18000 g. Once the resulting supernatant was transferred to a fresh tube, a 1x volume of iced isopropanol was added and DNA was precipitated during an overnight incubation at -20°C. The tubes were centrifuged at 18000 g for 20 minutes to pellet the DNA. Pelleted DNA was washed in 70% cold ethanol. The ethanol was removed by pipette and the pellets were allowed to air-dry for twenty to thirty minutes. The pellets were resuspended in 50 µL of Tris-EDTA buffer (10mM Tris, 1mM EDTA, pH 8.0; TE). To reduce lipid contamination and CTAB extraction carry-over, the suspended samples were cleaned using the Zymo DNA Clean & Concentrator Kit™ according to manufacturer's instructions (Appendix 2). Cleaned DNA was eluted in 30 µL of DNase-free water (Promega). Final DNA concentration in µg/µL was assessed with a NanoDrop Lite spectrophotometer (Thermo Scientific). DNA samples were then analyzed for the presence of endophyte DNA using PCR and gel electrophoresis.

PCR was conducted using ITS-based primer pairs for amplification (Appendix 3). These primers amplify conserved ribosomal DNA internal transcribed spacer (ITS) regions 1 and 2, as

well as the 5.8S gene. Primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used (WHITE *et al.* 1990). ITS1F was used instead of ITS1 (above) because it yielded longer and more distinct bands. The following PCR reaction mixture was used: 12.5 µL of 2X Promega™ PCR Master Mix, 0.5 µL of each primer, 1 µL of template DNA, and 10.5 µL of DNase-free water. Thirty-five PCR cycles were run in a C1000 Touch™ thermal cycler (Bio Rad) with the following reaction conditions: following an initial melt at 94°C (3 minutes), each cycle consisted of a denaturation step at 94°C (60 seconds), an annealing step at 54°C (2 minutes), and an extension step at 72°C (2 minutes). After the 35th cycle, a final 10-min extension step was performed at 72°C.

PCR products were separated in 1.0% agarose gels (Fermentas) in 1X TAE buffer (40mM Tris, 20mM Acetate and 1mM EDTA) with 0.35% µg/mL of ethidium bromide. Products with distinct bands were cleaned with a Zymo DNA Clean & Concentrator™ -5 kit to minimize PCR artifacts, quantified with a spectrophotometer (Nanodrop: Thermo Scientific), and adjusted to 5 µg/µL concentrations before being shipped on ice to GENEWIZ for sequencing.

The resulting sequence chromatograms showed a great deal of background, rather than clean nucleotide peaks, suggesting that multiple species were being amplified with each sample (Fig. 2). The sequences were analyzed in BLAST through the National Center for Biotechnology Information (NCBI. http://blast.ncbi.nlm.nih.gov/Blast.cgi). Initial sequences returned BLAST hits with 75 – 95% sequence homology, which is below standard reporting confidence (95% and above) in literature concerning molecular analysis of fungal endophytes. These results led us to hypothesize that any given PCR bands that were sequenced included genetic material from an endophyte community. This hypothesis was tested by cloning the PCR product and sequencing the inserts in the clones.

Cloning of PCR products

DNA isolated from needles sampled on March 15, 2015 was used for all cloning procedures. New PCR products were generated using primers ITS1-F and ITS4 (White, *et al*., 1990; PCR conditions were as follows: 35 cycles of 95°C, 3 minutes; 95°C, 30 seconds; 57°C, 2 minutes; 72°C, 2 minutes; and a final elongation step at 72°C for10 minutes). A TOPO TA® kit for Sequencing (ThermoFisher Scientific, K450002) was used to insert freshly amplified fragments into the plasmids provided and to transform chemically competent *E. coli* cells provided with the kit (Appendix 4). Transformed cells were grown on selective media (LB agar with 50µg/ml of ampicillin) to identify transformants. Individual distinct colonies that developed on the selective media were inoculated in LB broth with ampicillin (50µg/ml). Colonies were selected at random and each was transferred to 10mL of broth in a sterile glass test tube and shaken overnight at 200 rpm in an incubator-shaker at 37°C.

A Zyppy™ Plasmid Miniprep Kit (Zymo Research; D4019) was used to extract plasmids from cultured cells. Clean plasmid DNA was used as template in PCR reactions using the ITS primers to confirm insert size and samples producing clean bands were sent to GENEWIZ for sequencing. Most of those samples yielded clean sequence chromatograms with low background, and good quality sequence homology with BLAST (95 – 100%).

RESULTS

Needle plating

Sixteen plates were discarded due to yeast contamination (colonies growing on plates apart from needle sections). Endophyte emergence from sectioned needles increased from 35% (plated intact) to over 90% (compressed to increase surface area prior to sectioning), but only six distinct morphotypes emerged from nearly 500 recovered isolates. Four of those morphotypes emerged only from needles sampled at the Tyler location (3 from 2014 needles, and 1 from 2012

needles), and the Hog Lake location yielded one morphotype for each needle age. Only two isolates (both from Tyler 2014 needles) produced spores, and no identification was attempted.

Plasmid sequencing

Plasmid products extracted from 57 unique *E. coli* colonies containing an insert of the correct length were sequenced. Six plasmid samples yielded poor returns, and could not be compared with fungal sequences in NCBI BLAST. The BLAST results are summarized in Table 1. Of the remaining 51 sequences; 22 had identifiable taxonomic unit (ITU) assignments. Twenty-nine of the 51 sequences were highly homologous to previously published sequences that have not been assigned a taxonomic designation. Of these 51 isolates, 18% were common to either 2012 or 2014 needles at both Hog Lake and Tyler sites. Thirty six percent of the isolates were unique to Hog Lake needles, and 45% were unique to Tyler needles.

An endophyte lacking an ITU (an unidentified ascomycete; accession # JX136267) was isolated 12 times from both 2012 and 2014 needles at the Hog Lake site; while other sequences were isolated from 2012 or 2014 needles, but not both. There were three cloned sequences common to Tyler needles of both ages: sequenced clone JX136267 (isolated 4 times, in total), *Sporobolomyces gracilis* (isolated 4 times), and *Catenulostroma germanicum* (isolated 5 times). All other Tyler sequences were isolated from 2012 needles, or 2014 needles, but not both. Sequence JX136267 (common to needles of both years, at both sites) represented 31% of total sequences. Sequence JX136267 and the other ITUs occurring in needles of both ages together represented 53% of total sequences.

When we consider time of collection only, isolates found in both 2012 and 2014 needles numbered 14% of the total, while 41% were found only in 2012 needles, and 45% were found only in 2014 needles.

DISCUSSION

Throughout the course of this experiment, more than 4,000 needle segments were cultivated; while the numbers of needle sections showing some emergence were high (>90%), the diversity of emerging fungi was low. Those plates produced only six distinct isolates; of those, only two formed spores. Both sporulating isolates were ascomycetes, but neither was identified. Both the low diversity of endophytes emerging in plate culture and the lack of spore formation in two-thirds of distinct isolates necessitated a move to molecular techniques. Molecular techniques did, in fact, reveal a larger community then standard culturing. There were four times the diversity of isolates retrievable via DNA extraction and amplification as were cultivable in the lab. My results reinforce other work that shows that studies of endophyte communities relying upon plated culturing can drastically underestimate the diversity of such communities (RAJALA *et al.* 2013; RAO *et al.* 2013; TEJESVI *et al.* 2010). Some ITUs determined early in the project using PCR techniques without cloning were not retrieved when the project moved to cloning – this might suggest that earlier poor-homology "matches" in NCBI were artifacts of the PCR process, rather than real isolates. This study did not employ multiple primer sets amplifying short PCR amplicons, which may have decreased recovery error, but there is still the concern of amplifying chimeric sequences (RAO *et al.* 2013). More clone sequencing would assist in determining whether or not that was the case; however, budgetary constraints limited us to the amount of sequencing done here.

Several of the endophytes isolated in healthy needles are known plant pathogens, which is a finding that echoes previous work with cultured ponderosa endophytes (RIDOUT and NEWCOMBE 2015). *Taphrina* species are associated with peach, almond, and apricot leaf curls – they may grow as Ascomycetous yeasts, and then form asci for the purposes of moving from host to host. *Taphrina* species have not previously been associated with the healthy needles of ponderosa pine, but the needles may serve as an asymptomatic reservoir of this pathogen.

Dothidiomycetes are a large class of fungi containing both plant pathogens and decay fungi. *Didymella exitialis* is a leaf pathogen of wheat (CROMEY *et al.* 1994). Pucciniomycete species are plant rusts- *Endocronartium* (one of the genera within this subdivision), commonly infests ponderosa pine. Galls associated with this fungus were common throughout both study sites.

Ponderosa pine is subject to infection by a number of fungal blights (RIDOUT and NEWCOMBE 2015). In the winter and early spring of 2013 there was a multi-state bout of fungal needle drop in the west, the causative agent for which was not definitively identified. The infection caused browning and death of the outermost (nearly one year old) needles of ponderosa pine. As the warm season began, the browned 2013 needles dropped and left a stretch of naked branch in their wake; leaving a clean division between the 2012 and 2014 needles that were sampled in this experiment. Two of the ITUs to emerge from the molecular work belong to conifer pathogens. *Naemacyclus minor* causes disease in Scotch pine, and is a Christmas tree farm pest (causing needle drop). *Catenulostroma germanicum* is also associated with needle drop outbreaks, but does not have any reported association with disease in Ponderosa pine. In this study, it was isolated from asymptomatic needles at both sites and both years. The presence of potential blight fungi in asymptomatic leaves raises the question – is this an infection that exists at low levels all of the time, and just shows up periodically? Life histories of conifer blight fungi are not always well understood. Given the periodicity of needle blights, it seems possible that infection may arise from a hosted endophyte. Several *Lophodermium* species have been indicated in needle blight infections, and members of the genus are regularly isolated from pine (OONO *et al.* 2015), including ponderosa pine (RIDOUT and NEWCOMBE 2015). Might this be an endophyte that lives within the needle tissue all the time? Was either of the needle drop fungi isolated in this study (*Naemacyclus minor* and *Catenulostroma germanicum*) a contributor to the needle blight in 2013? This is not a determination that could be made, as the project commenced

after the infected needles had been dropped. If those needles had been collected and sampled before they were integrated into the forest floor, *and* the blight fungus could have been cultured in isolation in the lab, then Koch's postulates could have been followed to determine disease causation.

Although the two sites selected are only 5 km apart, they had only 18% ITUs in common. An examination of the endophytes hosted by *Cirsium arvense* and *Leucanthemum vulgare* (both in the family Asteraceae) showed no difference in communities along a 52 km transect (GANGE *et al.* 2007). Rhizomes of lesser galangal (*Alpinia offininarum*) sampled at five sites in China (the greatest distance between locations being over 1,900 km) also showed no difference in the endophyte communities hosted (SHUBIN *et al.* 2014). Loblolly pine (*Pinus taeda*) and Virginia pine (*Pinus virginiana*) sampled in the states of Virginia and North Carolina showed the endophyte *Lophodermium australe* in both pine species, and no differences in endophyte communities between sites. Conversely, cultured needle study of ponderosa pine endophytes just 85 km away from my sites showed a very different fungal community (RIDOUT and NEWCOMBE 2015). Whether the dissimilarity in endophyte communities in this study and Ridout and Newcombe's experimental forest study may be attributed to distance or difference in endophyte recovery approach could be addressed through direct extraction and DNA cloning from the trees sampled in the experimental forest.

The differences in endophyte communities between locations may be because the sites are located along a precipitation gradient. The Indian legume *Sesbania bispinosa* grows in sandy coastal dunes and mangroves: samples of the root endophytes in both ecosystems showed that more than 30% of the endophytes hosted were found in the roots of only one habitat (SHREELALITHA and SRIDHAR 2015). Native plants sampled along a 30 meter transect in an abrupt Canadian ecotone (aspen-dominated woodland to sphagnum-dominated wetland) showed

some endophytes common to the roots of five plants, but several that were specific to roots in wetland soil (WILSON *et al.* 2004). The Hog Lake site (though at a similar elevation), is slightly drier than the Tyler site; a variance that has contributed to a different composition of understory plants, and absence of ponderosa to the south and west of the Hog Lake site. Because the Hog Lake site borders an ecotone, it is also possible that its proximity to a biological community "edge" may contribute to increased species diversity (WALKER *et al.* 2003), or the selection for endophytic species that may lend enough stress tolerance to assist host trees in living near the limit of their natural habitat. A follow-up study might include a larger-scale sampling from sites at greater distances from one another, where both (or neither) border the same forest-to-sagebrush steppe transition. This modification would be helpful in determining whether endophytic community diversity between sites is consistent or partly attributable to site choice.

Observed endophyte diversity in needles of different ages supports previous work showing that older plant tissues have different communities of endophytes (CARROLL *et al.* 1977; FISHER and PETRINI 1992; STONE 1987). While the leaf study in asterids showed no significant difference in endophyte communities between locations, there was a positive correlation between the endophyte community size and plant height in *Leucanthemum vulgare* (GANGE *et al.* 2007). The lesser galangal study examined roots aged 1-4 years, and found a slight increase in species richness in older rhizomes (SHUBIN *et al.* 2014). As published works showing increased fungal species richness in older plant tissues tend to be culture-based, it would be worthwhile to verify whether molecular techniques demonstrate the same trend. New candles on ponderosa pine could be tagged and sampled, and the needles extracted held at -80°C until samples from the same bunches at 2, 3, and 4 years of age had been gathered. Direct extraction and cloning from those samples would produce a clear picture of the degree of endophytic succession in needles at those sites.

DNA samples for needle ages at each site were pooled for this study. A follow-up study should include a comparison of community diversity in needles of different ages in individual trees at each site.

Budget limitation prevented a larger-scale sequencing of cloned isolates for this experiment, but subsequent sampling and sequencing would assist in teasing out a more complete view of the needle endophyte community at large. Crucially, this molecular-based approach revealed a very different community than did the recent culture-based study examining the needles of ponderosa pine (RIDOUT and NEWCOMBE 2015). The only endophytic genus common to both ponderosa studies is *Hormonema*, which was identified to *H. dematioides* in Ridout and Newcombe's study, but was not identified to species in my own. *Hormonema* was isolated twice from the Tyler 2012 needles in this study, and several times in Ridout and Newcombe's (RIDOUT and NEWCOMBE 2015).

In conclusion, this study is the first to examine endophyte community diversity in *Pinus ponderosa* strictly by molecular means and lends further support to the idea that endophyte communities in general, and in ponderosa pine in particular, remain largely undescribed.

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FIGURES AND TABLES

Figure 1. Spokane County, WA. Tyler and Hog Lake sites.

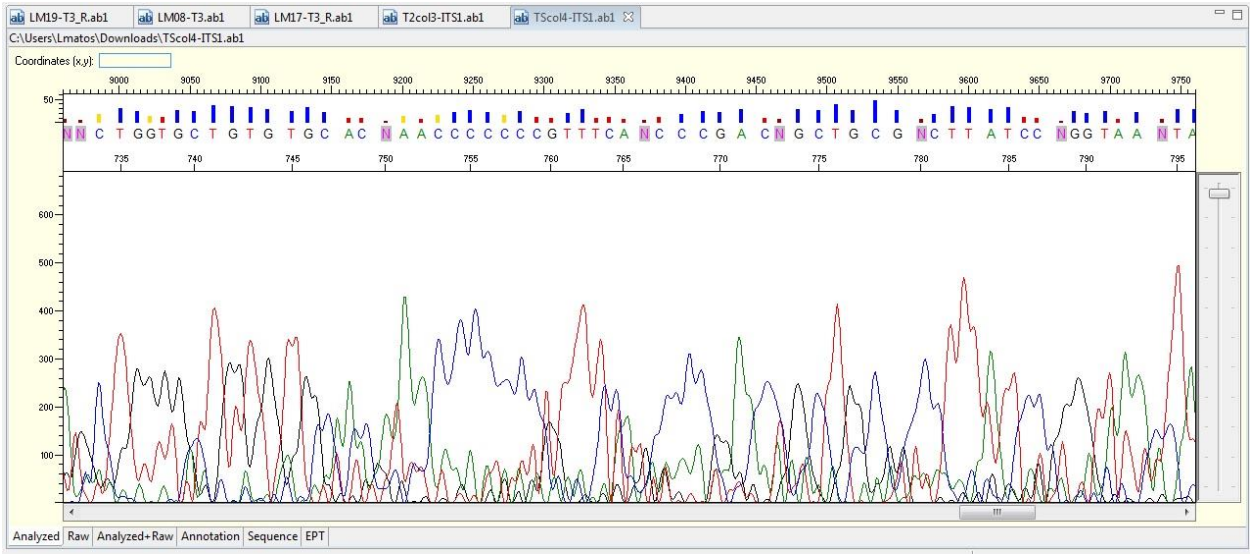

Figure 2. Noisy chromatogram resulting from sequencing a PCR band amplified from a hypothetical endophyte community within *Pinus ponderosa.*

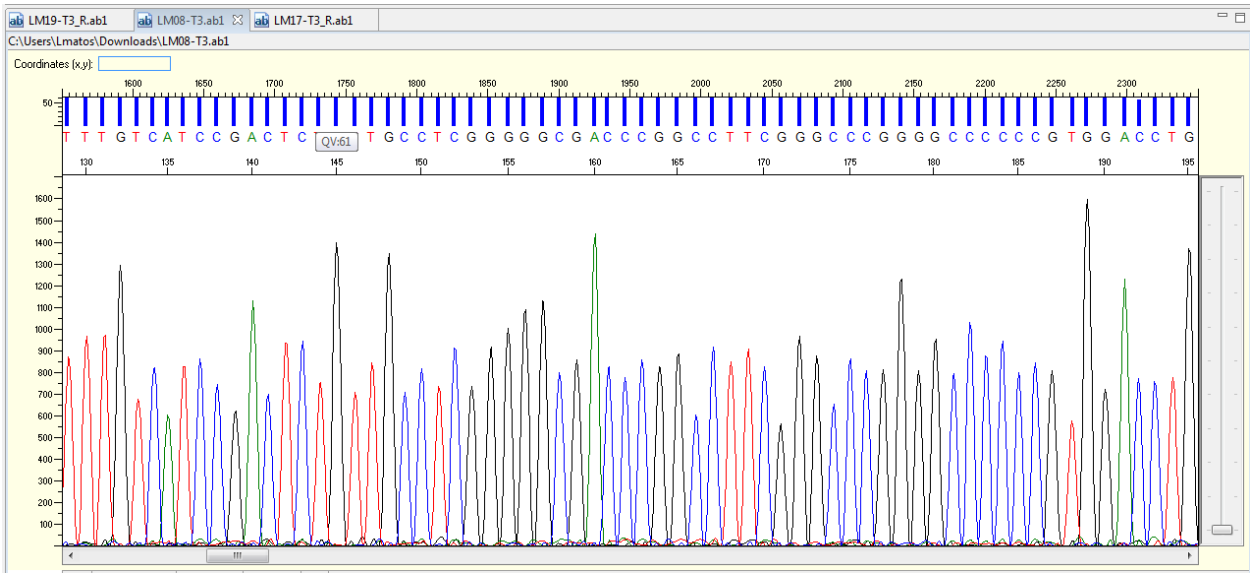

Figure 3. Non-noisy chromatogram resulting from sequencing plasmid inserts resulting from a procedure where the PCR band amplified from the hypothetical endophyte community was cloned and individual clone inserts were sequenced.

Table 1. Summary of BLAST results from sequenced clones. Cloned endophyte types with BLAST scores exceeding 90% for *Pinus ponderosa* needles. Fungal types with multiple accession numbers have BLAST scores with more than one identity.

Accession Number	Taxonomic Assignment	Homology (percent)	Base pairs	Hog Lake 2012	Hog Lake 2014	Tyler 2012	Tyler 2014
KJ606680 AFO13222	*Naemacyclus minor* / *Cyclaneusma minus*	**99**	**979**		X		
KF297112	Uncultured fungus clone	**95**	**1038**		X		
EU852371	Uncultured *Taphrina* clone	**95**	**1076**		X		
FR682186	Uncultured Ascomycete	**91**	**647**	X			
JX136265	Uncultured fungus clone	**98**	**532**	X			
KF800446	Uncultured fungus clone	**99**	**801**	X			
KF800339 AM901772	Uncultured fungus clone	**94**	**630+**	XX			
FJ553309 NR_121503	Uncultured Pezizomycotina / *Sarcinomyces crustaceous*	**99**	**791**	X			
JX136267 JX136424	Uncultured fungus clones	**98-99**	**597+**	XXXXX XXXX	XXX	XX	XX
EU019253	*Catenulostroma germanicum*	**98-99**	**696+**		X	X	XXXX
HM037657 GU174374	Uncultured fungus clone	**100**	**1049**		X		X
NR_073318 AB178481 AF189985	*Sporobolomyces gracilis*	**97**	**682+**	X		XX	XX
EU343283	*Teratophaeria microspora*	**98**	**976**				X
JF705944	Uncultured Pucciniomycete	**99**	**994**				X
AM901708	Uncultured Basidiomycete	**99**	**1032**				X
JX136334	Uncultured fungus clone	**97**	**592**				X
HF947037	Uncultured fungus clone	**95-99**	**630+**				XX
FJ553092	Uncultured Dothideomycete	**99**	**993**				X
AF013225	*Hormonema* ssp.	**96**	**900+**			XX	
JF749180 JQ759619	Ascomycota / Uncultured Dothideomycete	**99**	**879+**			XX	
KC753407 EU167564	Uncultured *Didymella* / *Didymella exitialis*	**100**	**974**			X	
JX136296 JX136411	Uncultured fungus clone	**99**	**739**			X	

Table 2. Host information for sequenced clones with strong matches in Genbank.

Accession Number	Taxonomic Assignment	Citation and Host Information
KJ606680 AFO13222	*Naemacyclus minor* / *Cyclaneusma minus*	**(JOHNSTON *et al.* 2014)** Monterey pine in New Zealand / Catal and Adams, 2004: direct submission.
KF297112	Uncultured fungus clone	**(TIMLING *et al.* 2014)** Soil core communities in the North American arctic.
EU852371	Uncultured *Taphrina* clone	**(BAKYS *et al.* 2009)** leaf and shoot bark isolate from European ash in Sweden.
FR682186	Uncultured Ascomycete	**(PITKARANTA *et al.* 2011)** Fungi of settled dust in renovated buildings in Finland.
JX136265	Uncultured fungus clone	**(HUFFMAN *et al.* 2013)** Bioaerosols and ice nuclei in *P. ponderosa* woodland forest, CO.
KF800446	Uncultured fungus clone	**(RITTENOUR *et al.* 2014)** Indoor bioaerosols in Kansas City.
KF800339 AM901772	Uncultured fungus clone	**(PITKARANTA *et al.* 2011; RITTENOUR *et al.* 2014)** Fungi of settled dust in renovated buildings in Finland.
FJ553309 NR_121503	Uncultured Pezizomycotina / *Sarcinomyces crustaceous*	**(HARTMANN *et al.* 2009)** Soil communities in lodgepole pine woodland of Skulow Lake, BC Canada **(TSUNEDA *et al.* 2011)** bark fungi of balsam poplar in Edmunton, Alberta, Canada.
JX136267 JX136424	Uncultured fungus clones	**(HUFFMAN *et al.* 2013)** Bioaerosols and ice nuclei in *P. ponderosa* woodland forest, CO.
EU019253	*Catenulostroma germanicum*	**(CROUS *et al.* 2007)**Taxonomic study, worldwide: isolated from stone (Germany).
HM037657 GU174374	Uncultured fungus clone	**(HANNULA *et al.* 2010)** Rhizosphere study around GM potato crops: Netherlands **(HASSETT and ZAK 2009)** Unpublished: Michigan hardwood forest microbe communities.
NR_073318 AB178481 AF189985	*Sporobolomyces gracilis*	**(SCORZETTI *et al.* 2002)** Systematics paper-sequence analysis of lab cultures from all over the world **(NAGAHAMA *et al.* 2006)** Phylogenetic study of lab strains: Japan.
EU343283	*Teratophaeria microspora*	**(CROUS *et al.* 2007)** Taxonomic study, worldwide: isolated from stone (Germany).
JF705944	Uncultured Pucciniomycete	**(LARKIN *et al.* 2012)** Western white pine forest, Montana, USA.
AM901708	Uncultured Basidiomycete	**(PITKARANTA *et al.* 2011)** Fungi of settled dust in renovated buildings in Finland.
JX136334	Uncultured fungus clone	**(HUFFMAN *et al.* 2013)** Bioaerosols and ice nuclei in *P. ponderosa* woodland forest, CO.
HF947037	Uncultured fungus clone	**(MOULAS *et al.* 2013)** Greenhouse study: Phyllospheric response to pesticides in pepper plants .
FJ553092	Uncultured Dothideomycete	**(HARTMANN *et al.* 2009)** Soil communities in lodgepole pine woodland of Skulow Lake, BC Canada.
AF013225	*Hormonema* ssp.	**(CROUS *et al.* 2007)** Taxonomic study, worldwide: isolated from stone (Germany).
JF749180 JQ759619	Ascomycota / Uncultured Dothideomycete	**(LARKIN *et al.* 2012)** Western white pine forest, Montana, USA.
KC753407 EU167564	Uncultured *Didymella* / *Didymella exitialis*	**(PITKARANTA *et al.* 2011)** Fungi of settled dust in renovated buildings in Finland.
JX136296 JX136411	Uncultured fungus clone	**(HUFFMAN *et al.* 2013)** Bioaerosols and ice nuclei in *P. ponderosa* woodland forest, CO.

APPENDICES

Appendix 1 - CTAB DNA Extraction Protocol

1. Make the CTAB stock solution. For 1 liter of CTAB buffer, assemble the following.
    a. 100 mL of Tris-Cl stock (1M) pH 8.0
    b. 280 mL of NaCl stock (5M)
    c. 40 mL EDTA stock (0.5M)
    d. 20 g CTAB powder (cetyltrimethylammoniumbromide)
    e. Combine, bring pH to 7.5 – 8.0, and bring to a 1 liter total volume with distilled water. Autoclave and store at room temperature.
2. Assemble the following
    a. CTAB stock solution
    b. Polyvinylpyrrolidone (PVP)
    c. beta-mercaptoethanol

Cetyltrimethylammoniumbromide (CTAB) working solution recipe.

CTAB	PVP	ß-mercaptoethanol
0.5 mL	0.02 g	2.5 µL
5 mL	0.2 g	25 µL
20 mL	0.8 g	100 µL

Notes: Do not add PVP or beta-mercaptoethanol until certain of the quantity needed. Once these have been added, the shelf life of the buffer is only 2-3 days. If extracting DNA from plants or small insects with a thick, waxy cuticle, samples may require freezing and grinding. If grinding, skip step 8. If bead-beating alone will suffice, skip steps 7.b. through 7.d.

3. Setup the following things

a. Turn on a heat block or water bath for test tubes and set it to 65°C. Fill the metal block insert half-way with dH_2O, leaving enough room for tube incubation.
b. Put isopropanol on ice.
c. Put 70% ethanol in the fridge (4°C).
d. Frozen cold block (4°C)
e. Fill each of your bead-beating 1.5 mL polyethylene screw-cap grinding vials with 25 (0.5 mm diameter) glass beads. Label sides and caps.
f. Make a working solution (Table 2) in a 50 mL polypropylene tube.
    i. Dissolve the PVP in the CTAB buffer
    ii. Add ß-mercaptoethanol
    iii. Mix the three components by gentle inversion

    Note: CTAB is a surfactant, so rough treatment will produce foam-like bubbles.

    iv.

4. DNA extraction
    a. Surface sterilize the samples
        i. Submerge samples in 70% ethanol for 30 seconds
        ii. Rinse samples with sterile water
        iii. Let samples air dry on sterile paper
    b. Add 600 µL of CTAB buffer to labeled polyethylene screw-cap grinding vials
    c. Put vials in the tabletop incubator to bring them to 65°C
    d. Add 250 mg or less of sample tissue to a sterile mortar
    e. Carefully add 2-3 mL of liquid nitrogen
    f. Immediately grind sample into a fine powder with a sterile pestle

Note: If working with 50 mg or more of sample, 3-4 more additions of liquid nitrogen may be necessary before one has achieved a fine consistency.

g. Quickly tap out the frozen powder onto weigh paper over an ice block
h. Use the paper to funnel pulverized sample into 65°C grinding vials (without letting the powder thaw until it hits the hot CTAB)
i. Cap tightly

   Note: Samples may sit upright on the bench for a few minutes while you prep other samples. Having several sterilized mortar and pestles at your disposal will streamline this process.
j. Add the tubes to a bead beater set at 3400 strokes per minute.
k. Beat samples three times in 20 bursts

   Note: at a time until samples have been homogenized for 1 minute. Make sure the grinding vial isn't leaking in between 20-second bouts. Do not leave samples unattended when the bead beater is in motion.
l. Place homogenized samples back into the 65°C incubator
m. Incubate tubes at 65° for 60 minutes
n. Centrifuge at 2000x *g* for 10 minutes (4°C).
o. Transfer the supernatant to a fresh, labeled tube.

   Note: Record the volume recovered. Discard tubes with beads and organic matter.
p. Incubate the supernatant at 37° for 30 minutes.
q. Add 1.5x volume 5 M NaCl and 1x volume chloroform:isoamyl alcohol (24:1) to each tube and mix by inversion
r. Centrifuge at 18000x *g* for 20 minutes (4°C)
s. Carefully remove the aqueous phase and transfer it to a new tube

t. Add 1x volume chloroform:isoamyl alcohol

u. Centrifuge again at 18000x *g* for 20 minutes (4°C)

v. Transfer aqueous phase to a fresh labeled tube

w. Add 1x volume iced isopropanol and let DNA precipitate **overnight** at -20°C.

x. Centrifuge at 18000x *g* for 30 minutes (4°C)

Note: DNA pellets should become visible at the bottom of the tube.

y. Carefully remove the isopropanol.

z. Wash the pellets

i. Add 1mL of 70% ethanol

ii. Mix tubes by inversion

iii. Centrufuge18000x *g* for 5 minutes (4°C)

iv. Remove ethanol

v. Quick spin (30 seconds at room temp) the tubes in a table top centrifuge

vi. Remove any remaining ethanol

vii. Air dry the pellets (15 minutes)

aa. Resuspend each pellet in 50µL sterile water or 50 µL of Tris-EDTA (TE) buffer

bb. Flick the capped tube until the pellet is suspended

cc. Determine final DNA concentration with a nucleic acid with a spectrophotometer or fluorometer

dd. Store DNA at -20°C

Appendix 2 – Zymo™ DNA Clean and Concentrator Kit Protocol

All centrifugation steps were performed at 16,000 x g at room temperature.

1. DNA binding
    a. To a 1.5 ml microcentrifuge tube with your sample
    b. Add 5 volumes of DNA Binding Buffer to each volume of DNA sample
    c. Mix briefly by vortexing
    d. Transfer mixture to a provided Zymo-Spin™ Column2 in a Collection Tube
    e. Centrifuge for 30 seconds. Discard the flow-through
2. DNA wash
    a. Add 200 µl DNA Wash Buffer to the column
    b. Centrifuge for 30 seconds. Repeat the wash step
3. DNA elution
    a. Add 15µl of DNAse-free water directly to the column matrix
    b. Incubate at room temperature for one minute
    c. Transfer the column to a 1.5 ml microcentrifuge tube
    d. Centrifuge for 30 seconds to elute the DNA
    e. Store the eluent at -20°C until use

Appendix 3 – PCR and Electrophoresis Protocols

Polymerase Chain Reaction

All PCR reactions were conducted using a general master mix (Promega, M7505). The thermal cycler used for all PCR reactions was a Biorad C1000 Touch.

The following conditions were used for PCR using ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC)

95°C for 3 min (initial denaturation)

35 cycles of

95°C for 1 min, denaturation

57°C for 2 min, annealing

72°C for 2 min, elongation

After the 35 cycles, a final extension step was done at 72°C for 10 min.

Gel Electrophoresis

Materials needed: Agarose

TAE Buffer

6X Sample Loading Buffer (New England Biolabs, B7021S)

2- Log DNA ladder, (New England Biolabs, N3200L)

DNA stain (ethidium bromide or other)

Recipe: 50X TAE Buffer STOCK

242 g Tris Base

57 ml Glacial Acetic Acid

100 ml 0.5M EDTA (pH 8.0)

Bring the total volume up to 1L with water

1. Making and pouring a 60 ml 1% agarose gel
2. Add 0.6g of agarose to 60 ml of 1X TAE Buffer in Erlenmeyer flask
3. Microwave for 1:30 min. and remove to swirl and observe
4. Look to see if there are still crystals in solution. If crystals are present, place in microwave for another 30 sec. or until fully dissolved.
    a. Add 5 µl of 1 mg/ml Ethidium Bromide to solution, swirling to mix
    b. Wait for solution to cool to 55-60°C or place Erlenmeyer flask on ice to speed up process.
        i. This temperature should be at about when the flask can be handled with bare hand (and glove).
    c. Place comb into casting tray into slots at the end
    d. Pour solution into casting tray and let set until firm (at least 40 minutes)
5. Loading the gel
    a. Gently remove comb by pulling straight up
    b. Move casting tray into gel box with wells towards the black (-) end and add sufficient 1X TAE Buffer to fill the wells and cover the gel with 5mm of buffer
    c. Load 1st well with 5µl of 2 Log ladder mixed with 5µl of TE
    d. Load the subsequent wells with the samples: mix 5µl of each sample with 5µl 10mM tris, 1mM EDTA, pH 8.0 (TE) and 2µl of loading dye
        i. Notes: Check to make sure that no air has been drawn into the pipette tip after the sample has been drawn up. Air at the very tip will become a bubble in the loading buffer, and "puff" the sample out of the well. The pipette tip need not be to deeply inserted into the well, or you run the risk of puncturing the bottom of the gel and losing much of your sample.

6. Running the gel
    a. Connect the gel box to the power supply
    b. Turn the power supply ON
    c. Set the range to LOW and the voltage to 90V (±2V)
    d. Run for 60 minutes
        i. Make sure that bubbles are visible rising from the bottom of the box. This indicates that the gel is running properly.
7. At the end of the run, carefully remove the casting tray and photograph the gel using a UV transilluminator.
8. Discard of the gel in the appropriate hazardous materials container.

Appendix 4 – Cloning and sequencing of PCR products

TOPO TA Cloning® Kit For Sequencing with pCR 4 TOPO (ThermoFisher Scientific, K450002)

Note: Leave chemically competent *E. coli* in storage at -80° until you are ready to transform cells.

1. To set up a cloning reaction, Assemble the following
    a. Fresh PCR product.
    b. An ice bath
    c. S.O.C. medium, warmed to room temp
    d. 42°C water bath
    e. 37°C shaking and non-shaking incubator
    f. Water at room temperature (provided with the TOPO TA Cloning® Kit)
    g. Thaw the salt solution and TOPO vector (pCR4) on ice.

2. Perform cloning reaction
    a. To 3 µl water in an autoclaved tube, add 1 µl fresh PCR product.
    b. Add 1 µl salt solution.
    c. Add 1 µl thawed TOPO vector (pCR4)

    Mix reaction gently, and incubate for 5 (or more – up to 30) minutes at room temperature (21°-25°C)

    Place the cloning reaction on ice.

3. Transform Top10 chemically-competent cells

    Note: Each transformation requires **one** vial of competent cells and **at least** two selective plates.

a. Warm selective plates (ampicillin or kanamycin) in the non-shaking incubator for 30 minutes.

b. Thaw **on ice** 1 vial of One Shot **(**TOP10) *E. coli* cells **only when you are ready to proceed**.

c. Add 2 µL of the cloning reaction into a vial of the One Shot **(**TOP10) cells and mix gently. DO NOT mix by pipetting up and down.

d. Incubate on ice for 5 – 30 minutes.

e. Heat-shock in the water bath at 42°C for 30 seconds without shaking.

f. Quickly transfer tubes to ice.

g. Add 250 µL room-temp S.O.C medium.

h. Cap the tube and shake horizontally @ 200 rpm at 37°C for 1 hour.

i. Spread 10-50 µL from each transformation on a pre-warmed plate. Add 20 µL of S.O.C. medium to insure even spreading if adding a small volume.

j. Incubate plates overnight at 37°C.

k. Several hundred colonies should be visible. Choose approximately 10 for analysis.

l. Make patch plates for the colonies chosen before proceeding with plasmid extractions. These plates can be incubated at 30°C to 37°C overnight, and then held at 4°C for several days.

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